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In Vitro Antagonistic Activity Evaluation of Lactic Acid Bacteria (LAB) Combined with Cellulase Enzyme Against *Campylobacter jejuni* Growth in Co-Culture

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The antibacterial effects of nine lactic acid bacteria (LAB) against *Campylobacter jejuni* were investigated by using agar gel diffusion and co-culture assays. Some differences were recorded between the inhibition effects measured with these two methods. Only two LAB, *Lb. pentosus* CWBI B78 and *E. faecium* THT, exhibited a clear anti-*Campylobacter* activity in co-culture assay with dehydrated poultry excreta mixed with ground straw (DPE/GS) as the only growth substrate source. It was observed that the supplementation of such medium with a cellulase A complex (Beldem S.A.) enhanced the antimicrobial effect of both LAB strains. The co-culture medium acidification and the *C. jejuni* were positively correlated with the cellulase A concentration. The antibacterial effect was characterized by the lactic acid production from the homofermentative *E. faecium* THT and the lactic and acetic acids production from the heterofermentative *Lb. pentosus* CWBI B78. The antagonistic properties of LAB strains and enzyme combination could be used in strategies aiming at the reduction of *Campylobacter* prevalence in the poultry production chain and consequently the risk of human infection.

Keywords: Co-culture, lactic acid bacteria, *Campylobacter*, cellulolytic enzymes, antagonistic activity, lactic acid, acetic acid

During the last decade, the alimentary toxi-infection due to *Campylobacter* has considerably increased worldwide [31]. At the same time, "organic" and "traditional" products have gained in popularity among consumers [41], and the increased production of such products will not solve food safety problems. The principal source and reservoir of the *Campylobacter* pathogen is actually poultry and derivate products [40], especially when chickens have access to the outdoors or pasture [9]. A Danish study showed that 100% of 22 organic broiler-flock samples were positive for *Campylobacter* spp. compared with 36.7% of 79 conventional broiler flock samples [14]. A recent epidemiologic study carried out in the southern part of Belgium for one year demonstrated that 100% of sampled free-range broiler flocks were positive for *Campylobacter* during the warm season [39]. *Campylobacter* species are ubiquitous enteric bacteria in the environment and in domestic animal. Thus, they are widely disseminated in the farm environment, and they can be concentrated in raw poultry meat. Because of its susceptibility to multiple environmental stresses (*e.g.*, temperature, relative humidity, and air composition), it is known that *Campylobacter* does not grow outside its host, but is able to survive in litter [17], in sewage [1], in biofilms formed in the water distribution systems of poultry houses [12], and for more than two weeks in feces [32]. The reduction of *Campylobacter* contamination at different levels of the poultry production chain, especially at the primary production level, should decrease the risk of human infection. The control of *Campylobacter* prevalence in broilers is possible but still remains a real challenge. Many different strategies have been developed with more or less success and have been recently reviewed by Lin [16] and Vandeplas *et al*. [37]. At present, the usual methods that have been implemented to prevent *Campylobacter* colonization and that have been admitted by sanitary authorities are based on strict biosecurity [Codex Alimentarius Commission, 4]. However, hygiene

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procedures are limited by uncontrollable environmental factors in organics flocks (*e.g.*, open-air range, wild birds, rodents, farm animals, *etc*.) and by the difficulty to strictly respect biosecurity rules in conventional flocks throughout the rearing stages. In addition, many complementary methods have been developed during the last decade. Acidification of the flock environment (litter, drink water, and feed) was based on the acid susceptibility of *Campylobacter* [36]. Other investigated strategies include vaccine, phage therapy, or antibiotic uses as growth promoters. However, the latter are completely prohibited by the EU authorities since 2006 [European Economic Community, 8]. However, biological controls, especially microbial competition, are the most investigated approaches [20, 37, 44]. The bacteria most studied belong to the lactic acid bacteria (LAB) group, which have beneficial effects on health and an important role in preserving foods and preventing food poisoning [18]. These bacteria are characterized by their potential to prevent adherence, establishment, replication, and/or virulence of specific enteropathogens [26]. Different antagonistic mechanisms have been proposed; pH decrease *via* organic acids production, nutrient competition with pathogens, and/or production of specific inhibitory compounds such as bacteriocins [28]. Thus, the ubiquitous LAB use in the chicken environment (chicken house, litter, pasture, *etc*.) could reduce the pathogens population and their horizontal transmission in the farm, and could promote animal health.

The primary aim of this research was therefore to establish the effect of some LAB combined with the cellulase A enzymatic complex on growth of the enteropathogen *Campylobacter jejuni*. The LAB survival in the chicken excreta and their antagonist activity potential could be enhanced by glycanase enzyme, as stated by Vandeplas *et al*. [38]. These authors showed that a combination between an enzyme of xylanase type and a *Lactobacillus* strain used as feed additive was able to reduce the *Salmonella* Typhimurium concentration in caeca of challenged chickens. Enzymes can hydrolyze non-digestible polysaccharides that can be specifically metabolized by LAB. In this study, combinations of LAB strains and a cellulase enzyme were tested in order to further develop anti-*Campylobacter* treatments to be spread on poultry litter and/or open-air ranges. Thus, the antagonistic activity of different LAB has been tested using the agar gel diffusion and co-culture batch techniques. Subsequently, the cellulase A enzymatic complex was combined with two anti-*Campylobacter* strains, *Lactobacillus pentosus* CWBI B78 and *Enterococcus faecium* THT, in order to assess its ability to improve the antimicrobial activity of both LAB in co-culture. The molecular mechanism involved in antimicrobial activity was investigated by monitoring pH changes and organic acids levels throughout the time of co-culture.

Materials and Methods

Bacterial Strains

Nine lactic acid bacteria (LAB), which were isolated from environmental samples of chicken farms, were tested in this study; two *Pediococcus pentosaceus* (CWBI B73 and CWBI B605), two *Enterococcus faecium* (THT and CWBI B411), two *Paenibacillus* spp. (CWBI B1070 and CWBI B60), *Weissella confusa* CWBI B902, and two *Lactobacillus (Lb. pentosus* CWBI B78 and *Lb. plantarum* CWBI B76) strains. All these strains were obtained from the Walloon Center for Industrial Biology culture collection (CWBI, Gembloux, Belgium), except for *E. faecium* THT, which was cordially provided by THT s.a. (Gembloux, Belgium); all were characterized phenotypically by using the API 50 CHL System (bioMerieux, Lyon, France) and genetically by 16S rDNA sequencing analysis. The LAB cultures were preserved onto Man, Rogosa, and Sharpe (MRS) agar (Biokar Diagnostic, Beauvais, France) and in cryopreservation tubes at −80°C. Two *C. jejuni* strains (LMG 6446 and CWBI B1444) and two *C. coli* strains (LMG 6440 and CWBI B1445) were used as indicator organisms. These strains were kept without antibiotics in Brucella agar and conserved at −80°C in broth medium containing 15% (w/v) glycerol (Merck, Darmstadt, Germany).

Lactic Acid Bacteria and *C. jejuni* Culture Preparation

The LAB inoculum was prepared by subculturing bacteria two times in MRS broth (10 ml), at 30°C. The cells were harvested by centrifuging at 14,000 ×*g* for 5 min, at the end of the log phase. The supernatant was removed and the cells were washed twice in PBS (0.1 M, pH 7.4). The cells were resuspended to a final OD_{560} value of approximately 0.1 in Brucella broth, and 0.5 ml of this diluted cell solution was added to an anaerobic flask containing 150 ml of Brucella broth.

Before conducting the agar dilution test or the co-cultures, *Campylobacter* isolates were streaked from the −80°C stock onto Campy-Cefex agar, without antibiotics, and allowed to grow for 40–48 h at 42°C under a microaerobic environment (5% O_2, 10% CO_2, 85% N_2). Subsequently, the inoculum was prepared by transferring typical *Campylobacter* colonies produced on this medium into 10 ml of Brucella broth kept under microaerophilic atmosphere for 48 h.

Assessment of the Inhibitory Activity

Agar diffusion test. The antimicrobial activity of LAB was screened by the colony-overlay method [29]. The method was modified as follows: 5 µl of an overnight culture of LAB was spotted on a fresh MRS plate buffered with sodium phosphate (0.2 M, pH 7). The inoculated agar dishes were incubated for 16 h at 30°C and then overlaid with 5 ml of Campy-Cefex soft agar (0.5% agar) supplemented by 8 mM of triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) and seeded with 0.5 ml of 48 h microanaerobic *Campylobacter* culture. A diameter clear zone of 10 mm or greater, extending laterally from the border of the LAB spot after 24 h of incubation at 42°C under microanaerobic condition, was recorded as positive inhibition.

Co-culture experiments. Dehydrated Poultry Excreta (DPE), from previous feeding trials with broilers housed in raised-floor battery cages with mesh grate floors mounted over excreta collection trays, were prepared by hot drying at 60°C for 48 h and finely ground using a cyclone mill (Cyclotec Sample Mill 1093, Höganäs, Sweden)

with a 2-mm mesh screen. The sample was added to a co-culture medium as a nitrogen supplement source. Ground Straw (GS), which was ground in a grinder Cyclotec 1093, was used as a carbon source in the co-culture medium.

The co-culture experiments were performed by inoculating the LAB strains with *C. jejuni* LMG 6446 in the same broth culture. The mixed strains were grown in DPE/GS medium containing DPE, 1% (w/v); GS, 1% (w/v); Tween 80, 500 µl, and peptone, 2 g/l. All ingredients were mixed thoroughly and autoclaved for 20 min at 121°C. A 250-ml anaerobic flask containing 150 ml of sterile medium was inoculated with 1 ml of *C. jejuni* 48-h culture (~10^7–10^8 CFU/ml) and 0.5 ml of an overnight LAB culture diluted 100-fold (~10^5–10^6 CFU/ml). The microaerobiotic conditions were obtained by flushing the flask with a gas mixture (10% CO_2, 5% O_2 and 85% N_2) through the medium in the anaerobic culture flask for 20 min. The co-culture flasks were placed on an orbital shaker (Gallenkamp Orbital Incubator; Sanyo, Pocklington, UK) set at 80 rpm for 100 h at 37°C. To avoid disturbing the flask microaerobic atmosphere, the samples were withdrawn every 24 h during the fermentation period.

Numeration of *C. jejuni* and the LAB strain were performed on co-culture samples. One ml of sample was serially diluted 10-fold in bacterial diluent (8.5 g/l NaCl, 1.0 g/l peptone, and 1 ml Tween 80, pH 7.0) and plated on appropriate media described as follows. *Campylobacter* enumeration was carried out by direct plating onto Campy-Cefex agar with antibiotics (33 mg/l cefeperazone and 0.2 g/l cycloheximide). Plates were incubated in jars under microaerobic conditions using an Anaerocult C Gas-Pak envelope (Merck, Darmstadt, Germany) at 42°C for 48 h. An enrichment step of 24 h, by incubating 1 ml of first dilution mix into 9 ml of Bolton broth (Oxoid, Basingstoke, UK) at 42°C under microaerbic conditions, was carried out in order to confirm the absence of *Campylobacter* in the samples or to confirm the presence of *Campylobacter* in samples harboring less than 100 CFU of *Campylobacter* per milliliter (detection limit of the direct method).

The LAB strains were quantified by plating onto MRS agar supplemented with 0.2% calcium carbonate, and incubating the plates overnight at 37°C. Each plating dilution was performed in triplicate for *Campylobacter* and LAB.

Co-culture: Enzyme effect. To study the enzyme effect on the LAB antagonist activity, the co-culture was supplemented with an enzymatic complex called cellulase A, kindly supplied by Beldem s.a. (Andenne, Belgium). This enzymatic system, extracted from *Trichoderma reesei*, contains three glycohydrolase activities [endoglucanase (E.C. 3.2.1.4), cellobiohydrolase (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21)] and was chosen for its ability to hydrolyze straw/litter cellulose in easily metabolizable compounds [15]. Cellulase A was diluted in PBS buffer at pH 6 and sterilized by using 0.2 µm Pall Acrodisc filter (Pall Life Sciences, St. Germain en Laye, France) before being added into the culture at concentrations ranging from 0 to 500 mg/ml. After *C. jejuni* and LAB co-inoculation in a micro-aerophilic atmosphere as described above, the co-culture flasks were incubated at 37°C for 72 h. *Campylobacter* and LAB were quantified every 24 h as described above.

Determination of Co-Culture pH and Lactate, Acetate, and Glucose Concentrations

Ten ml of co-culture sample was adjusted to pH 10 with 2 M NaOH, treated with 0.2 g of polyvinylpolypyrrolidone (Sigma, St. Louis, MO, USA), shook vigorously for 5 min, and subsequently filtered on Whatman No.1 filter (VWR International, West Chester, PA, USA). The filtrate was diluted two times before lactate and acetate concentrations were quantified by high-performance liquid chromatography (HPLC). The HPLC module consisted of a Waters 2690 Alliance System (Waters, Milford, MA, USA) fitted out with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA), a Waters 717 automatic injector (Waters, Milford, MA, USA), a Waters 600 MS pump and system controller (Waters, Milford, MA, USA), and a column oven at 40°C. The mobile phase was 0.003 N H_2SO_4 at 5% (v/v) CH_3CN with a flow rate of 0.6 ml/min. The internal standard was isocaproic acid (Sigma, St. Louis, MO, USA), and the compounds were detected by a Waters 486 UV–visible detector (Waters, Milford, MA, USA) at 210 nm. The final glucose content in the co-culture supernatant was measured using an enzymatic glucose assay kit (Megazyme K-GLUC, Wicklow, Ireland). The co-culture pH was determined directly on samples with a 3151 pH meter (WTW GMBH, Weilheim, Germany).

Chemical Analysis

Total neutral sugar content in the co-culture medium was quantified with the phenol–sulfuric acid method as described by Dubois *et al.* [7], whereas, the reducing sugar content was determined using the 3,5-dinitrosalicylic acid (DNS) method [22]. The total Kjeldahl nitrogen and nitrate-nitrogen concentrations in the DPE/GS medium were evaluated by the international standard method (ISO1871:1994) in comparison with Brucella broth supplemented or not with 250 mg/ml of cellulase A.

Statistical Analyses

Statistical analysis was conducted with the general linear models procedure (GLM) of SAS (SAS Institute Inc., Cary, NC, USA). The time course of LAB and *C. jejuni* in the co-culture was analyzed by two-way ANOVA, including the effect of the LAB strain (n=4), the sample time (n=5), and any interactions when appropriate. A one-way ANOVA was applied for analyzing the effect of enzyme concentration on LAB antagonist activity (enzyme concentration as fixed factor, n=5), and metabolic activities of LAB in the co-culture (sample time as fixed factor, n=4). Means were compared using the Newman–Keuls test [6] and considered significant at $P<0.05$. Correlations between the glucose and organic acid concentrations in the co-cultures were determined using the CORR procedure of SAS, with a parametric Pearson's test.

RESULTS

Estimation of the antimicrobial activity of LAB against *C. jejuni*/*coli*, determined with the agar spotting method, is presented in Table 1.

All the tested bacteria were active against *C. coli*, excepted for *Paenibacillus* strains, which revealed no consistent activity. Five isolates produced antimicrobial substances that were active against at least one of the *C. jejuni* strains used as indicators, and only three isolates (*P. pentosaceus* CWBI B73, *E. faecium* THT, and *Lb. pentosus* CWBI B78) against both *C. jejuni* strains (LMG 6446 and CWBI B1444). No clear inhibition zone was

Table 1. Antimicrobial activities of lactic acid bacteria selected against *Campylobacter* indicator strains[a].

Indicator strains / Tested strains	*C. jejuni*		*C. coli*	
	LMG 6446	CWBI B1444	LMG 6440	CWBI B1445
E. faecium THT	++	++	++	++
E. faecium CWBI B411	-	+	++	+++
Lb. plantarum CWBI B76	-	-	++	++
Lb. pentosus CWBI B78	++	+	+++	++++
P. pentosaceus CWBI B73	+	+	+++	++
P. pentosaceus CWBI B605	-	+	++	+
W. confusa CWBI B902	-	-	+++	++
Paenibacillus sp. CWBI B1070	-	-	+	+
Paenibacillus sp. CWBI B60	-	-	+	-

[a]The different scores try to reflect different degrees of growth inhibition; -, no inhibition; +, 10–12 mm inhibition zone; ++, 13–15 mm inhibition zone; +++, 16–18 mm inhibition zone; ++++, 19–20 mm inhibition zone. All indicator strains were assayed at least twice.

observed with *Paenibacillus* sp. CWBI B1070, *Paenibacillus* sp. CWBI B60, *W. confusa* CWBI B902, and *Lb. plantarum* CWBI B76 with both *C. jejuni* strains used as indicators.

P. pentosaceus CWBI B73, *E. faecium* THT, and *Lb. pentosus* CWBI B78 were selected for their antimicrobial effects against *C. coli* as well as against *C. jejuni* and were tested in co-culture with *C. jejuni* LMG 6446. *Paenibacillus* sp. CWBI B1070 was used as a negative reference strain, which does not inhibit the *Campylobacter* growth. For this experiment, a DPE/GS medium broth co-culture was developed. In comparison with Brucella broth, the analysis of the DPE/GS medium, reported in Table 2, showed a poor availability of 0.364 g/l reducing sugars in comparison with 8.847 g/l in Brucella broth, whereas the total sugar content was also 2.2 times less important in DPE/GS medium than in Brucella broth. The sugar composition difference between both culture media led to a decrease of the bacterial growth of approximately 1.5 to 2.0 $\log_{10}$ CFU/ml in DPE/GS medium (data not shown). On the other hand, no significant difference in nitrogen content was reported in total Kjeldahl nitrogen and ammonia nitrogen between DPE/GS medium and Brucella broth. Whereas the DPE/GS medium supplied with cellulase A (250 mg/ml) increased the reducing sugar and total sugar availabilities, no significant effect was recorded on the percentage of total nitrogen and ammonia nitrogen. Otherwise, it was observed that the *C. jejuni* LMG 6446 numeration after 48 h incubation at 37°C was not different in DPE/GS medium supplied or not with cellulase A. The *Campylobacter* cell concentration reached 7.80 and 7.11 $\log_{10}$ CFU/ml in DPE/GS medium supplied or not with enzyme and Brucella broth, respectively.

All the LAB strains were in stationary phase after 24 h in co-culture (Fig. 1A). With initial inocula of approximately 10^3–10^4 CFU/ml, the concentrations of *Lb. pentosus* CWBI B78, *P. pentosaceus* CWBI B73, and *E. faecium* THT, were maintained approximately at 10^8–10^9 CFU/ml from 24 h of fermentation until the end of experiment, whereas *Paenibacillus* sp. CWBI B1070 only reached 10^6–10^7 CFU/ml. During the first 24 h, pH reduction can be correlated with the log-phase of LAB on each co-culture (Fig. 1B). The highest pH reduction was observed in *E. faecium* THT and *Lb. pentosus* CWBI B78 co-cultures, reaching 5.8 at 24 h and 6.0 at 48 h, respectively. Changes in pH throughout the fermentation did not affect the LAB growth in each co-culture. The pH reduction during the first 24 h did not induce any decrease of *Campylobacter* concentration during this period (Fig. 1C). It was only after 24 h of fermentation that a slow but steady decline of *Campylobacter* population was observed in *E. faecium* THT and *Lb. pentosus* CWBI B78 co-cultures. No formal correlation could be established between the pH decrease and the *C. jejuni* inhibition in *E. faecium* THT and *Lb. pentosus* CWBI B78 co-cultures. The pH reduction was observed during the first 24 h of fermentation, whereas the

Table 2. Effects of sugar and nitrogen sources combined or not with cellulase A on *C. jejuni* growth after 48 h incubation.

	Reducing sugar	Total sugar	Total Kjeldahl nitrogen	Ammonia nitrogen	*C. jejuni* growth after 48 h
	Eq. [Glc] g/l	Eq. [Glc] g/l	%	%	Log_{10} CFU/ml
Brucella broth	8.84±0.47	9.41±0.84	7.73±0.26	0.64±0.05	7.11±0.48
DPE/GS	0.36±0.02	4.25±0.02	7.95±0.11	0.68±0.01	7.81±0.30
DPE/GS+enz.	0.67±0.02	5.15±0.53	8.00±0.15	0.71±0.01	7.82±0.40

DPG/GS: Dehydrated poultry excreta (1%, w/v) mixed with ground straw [1% (w/v)].
enz.: cellulase A (250 mg/ml).

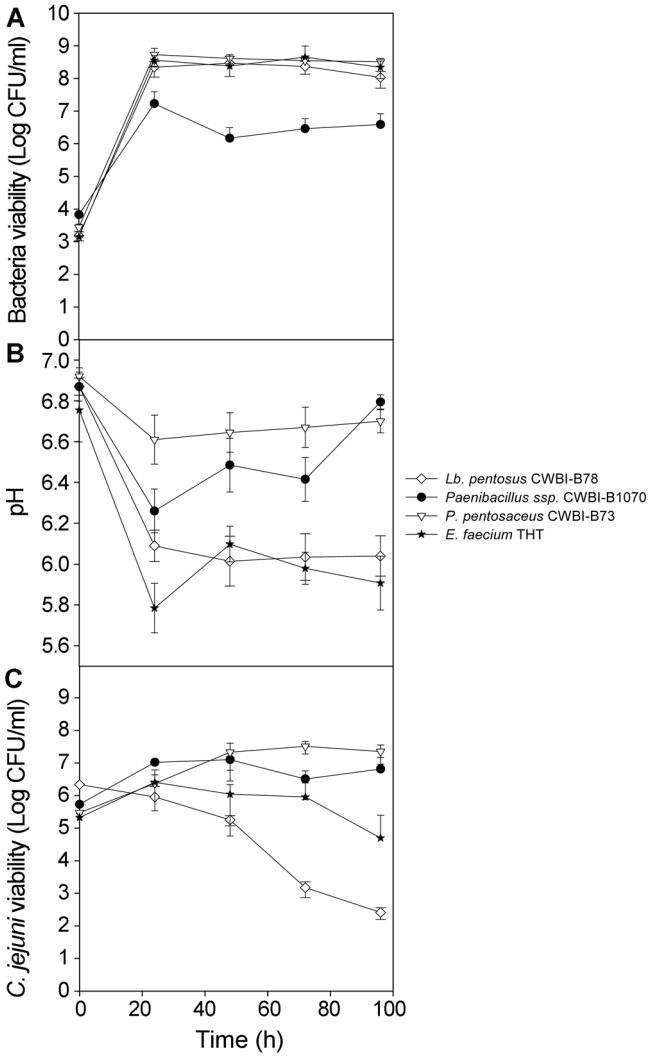

Fig. 1. Time course of lactic acid bacteria and *C. jejuni* in co-cultures.
(**A**) The viability of LAB; (**C**) *C. jejuni*; and (**B**) pH evolution in the co-cultures. The co-cultures were realized in DPE/GS broth medium under microaerophilic condition at 37°C, expressed as an average in log CFU/ml. Each value shown is the mean ± SD from at least two experiments.

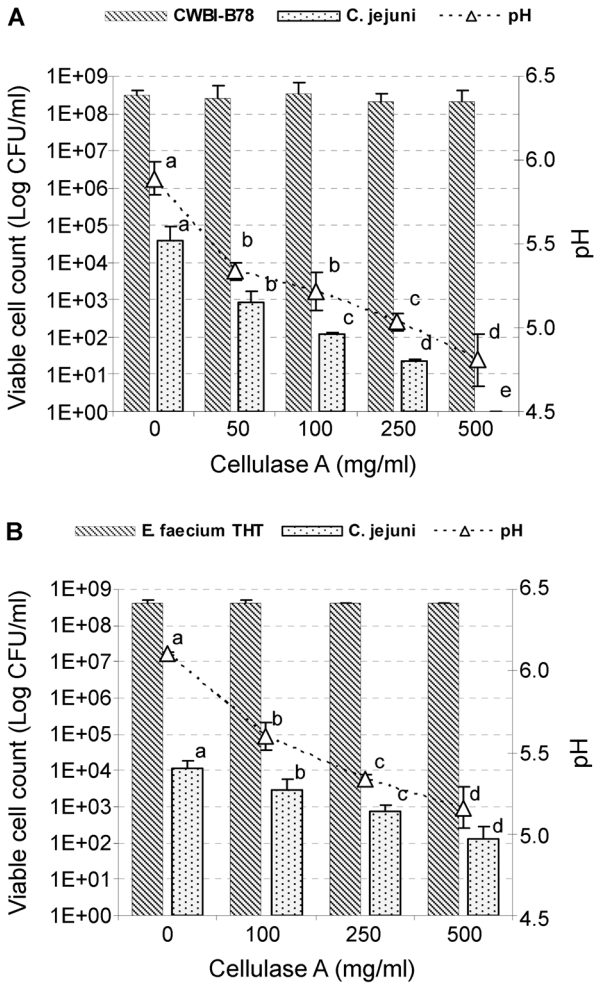

Fig. 2. Effect of enzyme concentration on LAB antagonist activity and pH in co-culture.
Several concentrations (0–500 mg/ml) of cellulase A enzyme complex added to co-culture *Lb. pentosus* CWBI B78+*C. jejuni* (**A**) and *E. faecium* THT+*C. jejuni* (**B**) Bacteria enumeration and pH in co-culture were determined at 72 h of incubation under microaerophilic condition at 37°C. The values are expressed as the mean ± SD from at least two sets of experiments. [a–e]Values with different letters at different enzyme concentrations differ significantly ($P<0.0001$).

pathogen concentration only began to decrease from 24 h. After 96 h of incubation, enteropathogens still survived at 4.6 log CFU/ml and 2.4 log CFU/ml in the bacterial co-culture of *E. faecium* THT and *Lb. pentosus* CWBI B78, respectively.

Cellulase A was added to the co-cultures in order to evaluate the possible impact of enzyme treatment on the antimicrobial capacity of LAB. The effect of cellulase A concentration on the inhibition potential was tested on *Lb. pentosus* CWBI B78 (Fig. 2A) and *E. faecium* THT (Fig. 2B) in DPE/GS medium. After 72 h of fermentation, the enzyme supplementation did not affect LAB growth. However, enzyme enhanced the acidification effect of both strains, which was positively correlated to the enzyme concentration. The pH variation between DPE/GS medium supplemented or not by 500 mg/ml of enzyme was statistically significant ($P<0.0001$) and reached 1.1 and 0.9 pH units in the *Lb. pentosus* CWBI B78 and *E. faecium* THT co-cultures, respectively. The decrease in pH was correlated ($P<0.0001$) to the reduction of *Campylobacter* population in broth fermentations. *C. jejuni* was reduced to an undetectable level after 72 h in *Lb. pentosus* CWBI B78 co-culture medium supplemented with 500 mg/ml of cellulase A (Fig. 2A). Under the same conditions with *E. faecium* THT, the pathogen concentration was stayed at ~10^2 CFU/ml. It appears, on the one hand, that the enzymatic complex had no bactericidal activity against LAB as well as against *C jejuni* (data not shown), but on

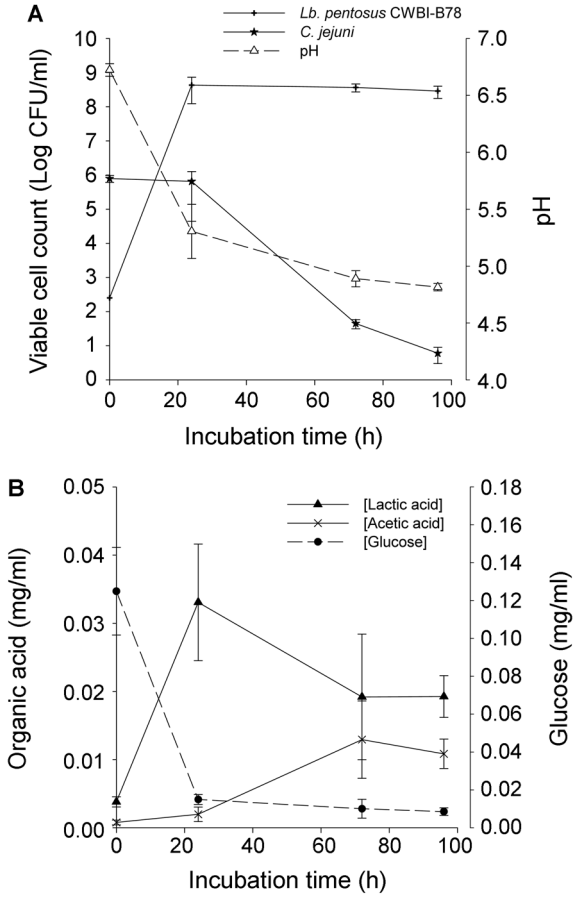

Fig. 3. Metabolic activity of *Lb. pentosus* CWBI B78 in co-culture.
(**A**) Growth curves of *Lb. pentosus* CWBI B78 and *C. jejuni* and pH evolution of the medium. (**B**) Time courses for the concentration of selected metabolites: glucose, lactic acid, and acetic acid. The co-culture was realized in DPE/GS broth supplemented with 250 mg/ml of cellulase A under microaerophilic condition at 37°C. Each value is the mean ± SD from at least two experiments.

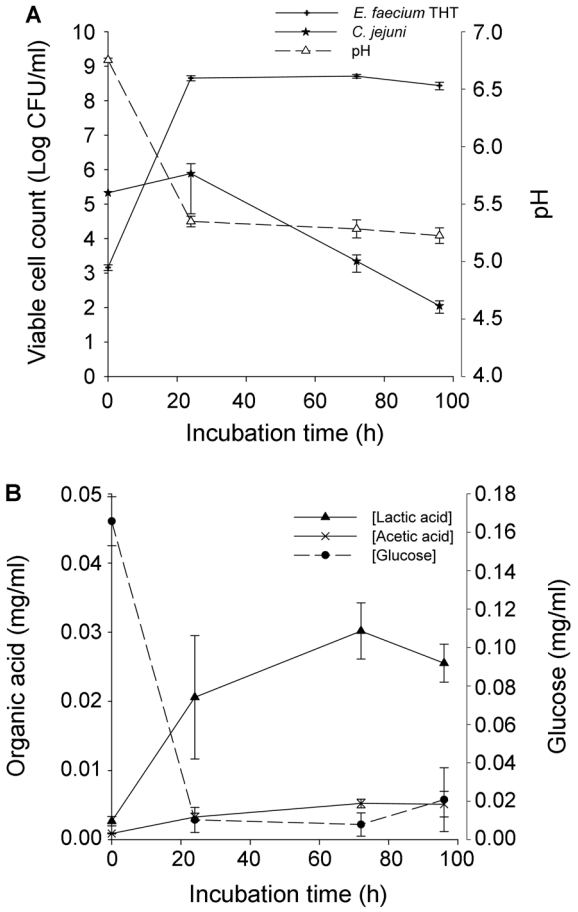

Fig. 4. Metabolic activity of *E. faecium* THT in co-culture.
(**A**) Growth curves of *E. faecium* THT and C. *jejuni* and pH evolution of the medium. (**B**) Time courses for the concentration of selected metabolites: glucose, lactic acid, and acetic acid. The co-culture was realized in DPE/GS broth supplemented with 250 mg/ml of cellulase A under microaerophilic condition at 37°C. Each value is the mean ± SD from at least two experiments.

the other hand it improved the LAB antagonism efficiency, probably by releasing oligosaccharides compounds metabolized by LAB but not by *Campylobacter,* which used amino acids or TCA cycle intermediates.

Organic acids and glucose concentrations were measured throughout the fermentation in *Lb. pentosus* CWBI B78 (Fig. 3) and *E. faecium* THT (Fig. 4) co-cultures supplemented with 250 mg/ml of cellulase A. The initial inocula were 2.4 and 3.1 log CFU/ml for *Lb. pentosus* CWBI B78 and *E. faecium* THT, respectively (Fig. 3A and 4A). After 24 h, the stationary phase for both LAB strains was reached, with a stable cell concentration of ~8.5 log CFU/ml until the end of the experiment. At the same period, the *Campylobacter* count in both LAB co-culture remained stable at ~5.8 log CFU/ml. When the antagonistic strains reached the stationary phase (~24 h) with a mean pH value of ~5.2, *C. jejuni* concentrations decreased in both co-cultures (Fig. 3A and 4A). In the *Lb. pentosus* CWBI B78 co-culture (Fig. 3A), the pH declined from about 5.2 after 24 h to 4.7 at the end of the incubation period, which corresponded to a pronounced decrease of *Campylobacter* numbers, which was below the limit of detection (2 log CFU) at 96 h. In the *E. faecium* THT co-culture (Fig. 4A), the pH value was stabilized at pH ~5.2 after 24 h; meanwhile, the *C. jejuni* population began to linearly decrease to reach ~2.2 log CFU/ml at 96 h.

The glucose concentration in both co-cultures was consumed in less than 24 h (Fig. 3B and 4B). At the same time, the lactate concentration increased proportionally ($P<0.009$) to glucose reduction, reaching concentrations of 33 and 21 mg/l in the *Lb. pentosus* CWBI B78 and *E. faecium* THT co-cultures, respectively. After 24 h of fermentation, the lactate concentration continued to increase up to 96 h, reaching 36 mg/l in the *E. faecium* THT co-culture (Fig. 4B). In contrast, the lactate concentration in the *Lb. pentosus* CWBI B78 co-culture was decreased at

the same time, reaching a final value of 19 mg/l (Fig. 3B). The lactate concentration was negatively correlated to the acetate content in the co-culture medium. The acetic acid concentration increased to reach 12 mg/l in *Lb. pentosus* CWBI B78, whereas in *E. faecium* THT it did not exceed 5 mg/l (Fig. 4B) after 96h of fermentation.

DISCUSSION

In the first part of this study, results of agar diffusion tests, which were used for estimating the antimicrobial activity of microorganisms from the poultry's environment, confirmed that some LAB have an antagonistic effect against *Campylobacter*. Most of the studies carried out with LAB strains belonging to the *Enterococcus* and *Lactobacillus* genera that were shown to inhibit *C. jejuni/coli* were performed *in vitro* using the agar gel diffusion test based on the Mayr–Harting method. This method uses culture supernatant from LAB fermentation performed in optimal growth conditions [20]. In this study, the agar gel diffusion method revealed that culture supernatant from *P. pentosaceus* CWBI B73, *Lb. pentosus* CWBI B78, and *E. faecium* THT can inhibit *C. jejuni* as well as *C. coli*. These bacterial species are known to produce antimicrobial molecules such as organic acids, fatty acids, ethanol, hydrogen peroxide, and bacteriocins, which have inhibitory effects on Gram positive [10] and negative [25] bacteria. Moreover, the inhibition ability of some LAB has also been demonstrated on Gram-negative bacteria, especially on *Campylobacter* growth [5, 23, 24]. In our experimental conditions, *Paenibacillus* sp. CWBI B1070 did not exert any anti-*Campylobacter* activity, in contrast to the results of Svetoch *et al*. [33], who reported antagonistic activities against *Campylobacter* of one *Bacillus* and three *Paenibacillus* strains isolated from the poultry production environment.

A co-culture method with a mixed DPE/GS medium was developed in this study and allowed us to directly observe the anti-*Campylobacter* effect of *Lb. pentosus* CWBI B78 and *E. faecium* THT in liquid medium. In comparison with the synthetic Brucella broth, the DPE/GS medium showed significantly different reducing sugars contents, whereas the nitrogen contents were similar. Nevertheless, it appears to be a culture medium as efficient as Brucella broth for co-cultures with *Campylobacter*. Gilpin *et al.* [11] have already reported that *Campylobacter* survive and grow in animal excreta. The co-culture technique allows to further appreciate the impact of LAB strains on the medium culture, on pH values, and on *Campylobacter* growth throughout the time course of the experiment. The results of *P. pentosaceus* CWBI B73 co-culture showed a weak pH decrease throughout the fermentation, which was related to the absence of *Campylobacter* reduction, contrary to the results from the agar diffusion method. This strain did not seem to be adapted to the less favorable conditions of the DPE/GS medium. This experiment also confirmed the inability of *Paenibacillus* sp. CWBI B1070 to be antagonistic against *Campylobacter*.

The use of exogenous enzymes is a common practice in modern biotechnology industries for biomass valorization, environmental sanitation, and food [42]. Cellulase and hemicellulase enzymes are largely involved in agricultural processes optimization, waste treatment [2], and improvement of feed efficiency and food fermentation [3]. These enzymes hydrolyze the dominant polysaccharides in plant cell wall to oligo/monomers. We hypothesized that these oligo/monomers could be specifically used by LAB strains for growth and metabolic activities. At this stage, *Lb. pentosus* CWBI B78 and *E. faecium* THT were the strains under investigation because of their strong inhibition activity against *C. jejuni* observed with the agar diffusion tests (Table 1). The complementation of the complex enzyme called cellulase A to the DPE/GS medium significantly enhanced the anti-*Campylobacter* activity of both strains (Fig. 2, 3, and 4). The antagonist capacity of both strains was increased proportionally to the increase of enzyme concentration, confirming the cooperation between celluase A and LAB. These results support the hypothesis that this enzyme could saccharify the cellulosic residues from the DPE/GS medium to more easily fermentable carbon sources. The enzyme use during composting of poultry litter has already been reported by Tiquia *et al*. [34], who demonstrated that an enzyme consortium (proteases, phosphatases, aminopeptidases, and glycosyl hydrolases) eliminated the fecal coliform population. In the same way, Logan and Bartlett [19] claimed that an enzyme–bacteria combination could act, like competitive exclusion [30], by reducing opportunistic microbes in favor of the bacteria of that present in the complex. Exogenous supplementation of glycohydrolases like cellulase A may support the bacteria, especially during the adaptation or the lag phase. This effect can be explained by enhancing microbial adhesion to a saccharide substrate, which makes substrate capture easier [35].

The antimicrobial activity that was observed in co-culture was investigated in the second part of this study in order to elucidate the underlying molecular mechanism of LAB–enzyme antagonism. During the first 24 h after inoculation of both *Lb. pentosus* CWBI B78 and *E. faecium* THT co-cultures, it was observed that the weak *Campylobacter* population reduction was not correlated with a pH decrease. The *Campylobacter* population began to decrease significantly once the culture pH reached its lowest value, after 24 h of culture. It appears that a pH diminution is a prerequisite to trigger the antimicrobial activity of the tested LAB strains. However, the results of lactic and acetic acid concentrations, which were measured by HPLC in co-cultures, suggest that production and high

concentration in short-chain fatty acids (SCFA) may directly induce *Campylobacter* inhibition. Indeed, lactate and acetate are major metabolic end products from LAB strain fermentation [28], and their toxicity at low pH has been explained by transmembrane flux of undissociated SCFA, which ionize in the cell, yielding protons that acidify the alkaline interior of the cell [27]. The time-course fermentation profile of *Lb. pentosus* CWBI B78 clearly showed that this strain produced preferentially lactate during the first hours (2–24 h) until glucose is consumed. Lactate concentration decreased while acetate was produced after 24 h of incubation (Fig. 3), probably by shift of the metabolic flux of the hexose monophosphate to the pentose phosphate [43]. The advantage of such of facultative heterofermentative strain is to produce acetate, which has more inhibitory potential against *Campylobacter* than lactate because of its higher pKa value [13]. Moreover, the co-culture supplementation with the cellulase A complex enhanced the antimicrobial activity of *Lb. pentosus* CWBI B78, which can ferment hexoses as well as pentoses released from the DPE/GS medium. On the other hand, *E. faecium* is an obligate homofermentative strain, which used hexoses to produce exclusively lactic acid, so its bactericidal effect is more limited (Fig. 2B and 4).

In conclusion, the results of this study demonstrated the antibacterial effect of *Lb. pentosus* CWBI B78 as well as *E. faecium* THT against *C. jejuni,* by the means of co-culture techniques and agar diffusion tests. The antagonism was enhanced in co-culture by supplementation of the medium, which was based on dry poultry excreta and ground straw, with the cellulase A complex. This apparent synergistic effect is consistent with observations of other authors with other pathogens. The co-culture model seems consequently to be more relevant than the agar gel diffusion technique to evaluate microbial interactions between an antagonistic strain and *C. jejuni* in a complex ecosystem like poultry litter. It has been pointed out that the inhibitory mechanism underlying this antagonistic effect is the ability of LAB to produce organic acids such as lactic and acetic acids. This work suggests that application of LAB in combination with a cellulase-type in environmental poultry could be used as a method for reducing *Campylobacter* populations, which could help to lower the risk of contamination in the poultry production industry. This hypothesis needs to be checked by developing an environmental treatment that could be spread on the poultry litter and on the open-air range and by testing in a free-range chicken experiment.

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